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Evaluation of selected binding domains for the analysis of ubiquitinated proteomes

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Abstract

Ubiquitination is an abundant post-translational modification that consists of covalent attachment of ubiquitin to lysine residues or the N-terminus of proteins. Mono and polyubiquitination have been shown to be involved in many critical eukaryotic cellular functions and are often disrupted by intracellular bacterial pathogens. Affinity enrichment of ubiquitinated proteins enables global analysis of this key modification. In this context, the use of ubiquitin-binding domains is a promising, but relatively unexplored alternative to more broadly used immunoaffinity or tagged affinity enrichment methods. In this study, we evaluated the application of eight ubiquitin-binding domains that have differing affinities for ubiquitination states. Small-scale proteomics analysis identified ~200 ubiquitinated protein candidates per ubiquitin-binding domain pull-down experiment. Results from subsequent Western blot analyses that employed anti-ubiquitin or monoclonal antibodies against polyubiquitination at lysine 48 and 63 suggest that ubiquitin-binding domains from Dsk2 and ubiquilin-1 have the broadest specificity in that they captured most types of ubiquitination, whereas the binding domain from NBR1 was more selective to polyubiquitination. These data demonstrate that with optimized purification conditions, ubiquitin-binding domains can be an alternative tool for proteomic applications. This approach is especially promising for the analysis of tissues or cells resistant to transfection, of which the overexpression of tagged ubiquitin is a major hurdle.

Keywords

Ubiquitination; post-translation modification; affinity purification; proteomics; mass spectrometry

Introduction

Ubiquitination is a reversible post-translational modification that consists of covalent attachment of ubiquitin, a 76 amino acid residue polypeptide to lysine residues or the N-terminus of proteins [1]. Importantly, this modification has been associated with many critical eukaryotic cellular functions such as protein degradation, autophagy of organelles, cellular trafficking, DNA damage repair, gene expression regulation, and signaling transduction [1, 2]. Protein ubiquitination requires coordinated action among the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and ubiquitin ligase E3; however, it is the latter enzyme that has the selectivity and specificity to target proteins [1]. Due to the great variety of E3 ligases it is expected that many proteins to be modified with ubiquitin, and not surprisingly, recent publications have identified several thousands of

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ubiquitination sites in cells [3-5]. Ubiquitination is also a reversible modification and the removal of ubiquitin units is catalyzed by specific proteases called deubiquinating enzymes or deubiquitinases [6, 7]. In addition to modifying other proteins, ubiquitin can form polyubiquitin chains, i.e., polymers of ubiquitin. These chains can be transferred in a similar fashion as single ubiquitin units to specific substrates, or remain in a free or unanchored form [1, 2]. While monoubiquitination is typically associated with cellular trafficking and gene expression regulation [2], polyubiquitination functionality depends on which ubiquitin lysine residue (K6, K11, K27, K29, K33, K48 and K63) or N-terminus serves as the linkage site of polymerization. Arguably, the best studied form is polyubiquitination at K48, which targets proteins to be degraded by the proteasome, although other polyubiquitination linkage forms have been associated with this function [8]. Other examples include linear (N-terminus linkage) and K63 polyubiquitination that have been shown to participate in cell signaling events [9, 10], and K11 that plays an important role in cell division [11].

Although the Gram-negative bacteria lack ubiquitination machineries, many pathogenic bacteria have in their genome genes encoding ubiquitin ligases and deubiquitinases. These gene products are secreted into the host cell cytosol and target several ubiquitination pathways [12-16]. Even though, several biological functions have been described for the bacterial ubiquitin ligases and deubiquitinases, their substrates are widely unknown. Thus a simple and versatile method for large-scale identification and fast validation of these substrates are highly desirable.

For global analysis of the ubiquitinated proteome, so far, the most used method for studying ubiquitinated proteins involves expressing a polyhistidine tagged form of ubiquitin, followed by affinity purification using immobilized metal (Ni^{2+} or Co^{2+}) affinity chromatography (IMAC) [17]. Although this is a straightforward procedure, it is limited to cell cultures and cannot be used to analyze animal tissues, human biopsies, and cells that are resistant to the uptake of exogenous recombinant DNA. In addition overexpression of ubiquitin can alter some cellular functions, as it was reported for p53/MDM2 pathway [18]. Alternatively, anti-ubiquitin polyclonal antibodies have been successfully applied to enrich ubiquitinated proteins [9, 19, 20]. However, a limitation of this approach is the undesired binding of antibody-interacting proteins along with a high background of antibody proteins in downstream analytical methods such as LC-MS/MS. Recently the development of monoclonal antibodies for specific polyubiquitination linkages has enabled the study of specific chains [9, 11]. Elsewhere, Xu et al. used antibodies against the signature diglycine residues that are remnants of ubiquitin after trypsin digestion to study global profiles of ubiquitination in a human cell line [21]. This method has been used in several papers so far [3, 22, 23], but it has a small limitation that the same signature diglycine residues are found in other ubiquitin-like protein, such as, NEDD8 and ISG15, making impossible to distinguish between these three PTMs.

The use of ubiquitin-binding domains (UBDs) is another approach to analyzing ubiquitinated proteomes. To date, more than 16 distinct types of UBDs have been described with a variety of functions and structures that are useful for capturing ubiquitinated proteins. UBDs are typically limited in length, i.e., ~20 to 150 amino acid residues [24, 25], which makes them highly desirable for proteomics research since their resulting peptides after trypsin digestion should lead to limited and known background species in mass spectrometry (MS)-based analyses. In other words, on-bead digestion of UBDs generates only a limited set of known relatively abundant peptides, but the in case of immunoaffinity, hundreds of peptides including those of unknown sequence, from the immunoglobulin variable region, are derived from the digestion of antibodies, increasing the background complexity. As some of the UBDs, such as ubiquitin-associated (UBA) domains of ubiquitin 1 (UQ1) and Dsk2 protein bind with similar affinities to many different linkages of polyubiquitinations

[26], their use potentially affords more complete characterization of the ubiquitination landscape. On the other hand, some UBDs may bind with higher affinity to specific types of polyubiquitination, as measured by surface plasmon resonance experiments with di- and tetra-ubiquitin chains [26]. Thus, UBDs could be used to perform more selective enrichment experiments [26]. Furthermore, the use of multiple UBDs in tandem, referred to as tandem ubiquitin-binding entities (TUBE) may greatly enhance the avidity to ubiquitinated proteins [27-29]. Despite their potential utility, there has been skepticism regarding the use of UBDs for global ubiquitinated proteomic analysis as most of these domains have low affinity to their targets [26, 30]. To date, few studies have demonstrated the use of UBD-based affinity purification for the analysis of ubiquitinated proteomes [31, 32], although a more systematic comparison between UBDs and optimization of the method has not been done yet.

In this study, we compared the performance of eight distinct UBDs for purifying ubiquitinated proteins from a complex cell lysate. A commonly used murine macrophage model cell line was chosen because of the importance of ubiquitination in signaling transduction during inflammation and because these cells are resistant to transfection, which undermines the use of an epitope tagged version of ubiquitin for affinity purification. Our study demonstrates a promising application of UBDs for global proteomic analysis, and highlights the relative advantages and limitations of this method.

Experimental

Materials and reagents

Eight UBDs conjugated to agarose were purchased from Enzo Life Sciences (Plymouth Meeting, PA): 1) p62-derived UBA, 2) hHR23B-derived UBA2, 3) NBR1-derived UBA, 4) NUB1-derived UBA, 5) UQ1-derived UBA, 6) Dsk2-derived UBA, 7) 19S proteasome subunit S5a-derived UIM, and 8) VPS9-derived CUE domains. K63 polyubiquitin-specific (monoclonal, Apu3), K48 polyubiquitin-specific (monoclonal, Apu2), anti-ubiquitin (polyclonal, AB1690) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (polyclonal, P342P) were obtained from Millipore (Temecula, CA). Polyvinylidene fluoride membranes and 4-20% SDS-PAGE gels were acquired from Invitrogen (Carlsbad, CA) and Bio-Rad (Hercules, CA), respectively. ECL blocking reagent was purchased from GE Healthcare (Piscataway, NJ). Salts, reagents, cyanogen bromide-activated agarose beads, and strong cation-exchange cartridges (50 mg, 1 mL) were obtained from Sigma-Aldrich (Saint Louis, MO). Silver stain and bicinchoninic acid kits, West Pico ECL reagent, and protease inhibitor cocktail (Cat # 78430) were purchased from Thermo Fisher Scientific.

Preparation of control beads

Control beads were prepared by incubating cyanogen bromide-activated agarose beads with 200 mM glycine (pH 8.0) overnight at 4 °C. After incubation, beads were extensively washed with 0.1 M NaHCO₃ (pH 8.0) containing 500 mM NaCl, and with 0.1 M sodium acetate (pH 8.0) containing 500 mM NaCl. Beads were subsequently stored at 4 °C in phosphate-buffered saline solution supplemented with 0.2% sodium azide.

Affinity purification

The RAW 264.7 murine macrophage cell line was purchased from American Type Culture Collection (Manassas, VA) and kept in Dulbecco's modified eagle's medium (American Type Culture Collection) supplemented with 10% heat-inactivated fetal calf serum, and streptomycin, and penicillin. Cells were maintained at 37 °C under 5% CO₂ atmosphere. Confluent cell cultures were washed twice with phosphate-buffered saline solution and scraped in 5 mL of lysis buffer [50 mM HEPES, 5 mM EDTA (pH 7.5) containing 150 mM

NaCl and 1% Triton X-100] with protease inhibitor cocktail and 5 mM iodoacetamide. Cells were then lysed in a sonoreactor (UTR200, Hielscher) for 3×30 s at 100% amplitude and 0.5 pulse. The extract was cleared by centrifuging for 20 min at $16,000 \times g$ and 4°C . Protein content was then measured using a bicinchoninic acid kit. Fifteen microliters (15 μL) of UBD-conjugated or control agarose beads were added to each milligram of protein and the samples were rotated overnight at 4°C . The beads were then washed twice with 20 volumes of each of the following solutions: lysis buffer, lysis buffer with higher salt content [50 mM HEPES, 5 mM EDTA (pH 7.5) containing 500 mM NaCl and 1% Triton X-100] and 100 mM NH_4HCO_3 (pH 7.5). Each wash was performed by rotating the beads for 5 min at room temperature and centrifuging for 5 min at $1000 \times g$. The samples were either submitted to on-bead protein digestion or eluted by adding one volume of SDS-PAGE sample buffer and incubating for 5 min at 95°C for Western blot analysis.

SDS-PAGE and Western blot analyses

Affinity purified proteins from an equivalent of 1 mg of cell lysate were separated in 4-20% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes, or silver stained according to the manufacturer's instructions. For the Western blot, the membrane was blocked 2% ECL blocking agent dissolved in TBS-T [20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween-20] and incubated overnight with either 500- (Apub3) or 1000-fold (Apub2 and anti-ubiquitin) dilutions of primary antibodies. The membrane was washed three times for 10 min each with TBS-T and incubated for 3 h with a 1000-fold dilution of secondary antibodies. Following incubation, the membrane was washed three times, and the reaction was developed with West Pico ECL reagent (Thermo) and visualized in a FluorChem Q imaging system (Alpha Innotech).

Protein digestion

Affinity-purified proteins obtained from duplicate pull-downs of cell lysate (equivalent to 0.5 mg total protein) were subjected to on-bead digestion. The beads were suspended in 200 μL 100 mM NH_4HCO_3 (pH 8.0) containing 5 mM DTT and incubated with agitation for 15 min at 55°C . The alkylation was performed by adding iodoacetamide to a final concentration of 10 mM and incubating for 30 min at 37°C with shaking at 600 rpm. The reaction was then diluted by adding 1 mL 25 mM NH_4HCO_3 and digested for 3h at 37°C with 1 μg trypsin. Digested peptides were recovered by centrifuging the beads 5 min at $1000 \times g$ and collecting the supernatant. The beads were extracted once more using 600 μL 10 mM ammonium formate (pH 3.0) in 25% acetonitrile (ACN). Both extracts were pooled together and sample pH was adjusted to 3.0 by adding 20% formic acid (FA) solution. The samples were cleaned in strong cation-exchange cartridges (50 mg resin, 1 mL, Supelco), using a SPE station (GX-274, Gilson). The cartridges were washed extensively with methanol, 10 mM ammonium formate (pH 3.0) in 25% acetonitrile (ACN), 500 mM ammonium formate (pH 6.8) in 25% ACN, and water, before being equilibrated with 10 mM ammonium formate (pH 3.0) in 25% ACN. After loading the samples, cartridges were washed with 10 mM ammonium formate (pH 3.0) in 25% ACN, and the peptides were eluted with methanol:water: NH_4OH (80:15:5, v:v:v) and concentrated in a vacuum centrifuge.

Liquid chromatography-tandem mass spectrometry analysis

Peptides were loaded onto capillary columns (75 $\mu\text{m} \times 65$ cm, Polymicro) packed with C18 beads (3 μm particles, Phenomenex) connected to a custom-made 4-column liquid chromatography LC system [33]. The elution was performed in an exponential gradient from 0-100% solvent B (solvent A: 0.1% FA; solvent B: 90% ACN/0.1% FA) with a constant pressure of 10,000 psi and flow rate of ~ 400 nL/min.[33] Eluted peptides were analyzed online in a linear ion-trap mass spectrometer (LTQ XL, Thermo Scientific, San Jose, CA).

Peptides were measured over a 400-2000 m/z range and the 10 most intense ions were selected for collision-induced dissociation (isolation width of 3 Da and 35% normalized collision energy). Dynamic exclusion was enabled to fragment each ion once before exclusion for 60 s.

Data analysis

LC-MS/MS spectra were converted into DTA files using default parameters, and submitted to SEQUEST (v27.12) [34] searches against the *Mus musculus* Ensembl database (23,088 sequences, downloaded from www.ensembl.org in December, 2010) and 186 common contaminant sequences (downloaded from www.ncbi.nlm.nih.gov/protein in August, 2006). All sequences were searched in both forward and reverse orientations (i.e., a total of 46,548 searched sequences). Parameters employed for searches were: 1) 3 Da and 1 Da for peptide and fragment mass tolerance, respectively; 2) partial tryptic digestion; 3) maximum of two missed cleavage sites; and 4) cysteine carbamidomethylation (+ 57 Da) and diglycine-lysine (+ 114 Da) as static and variable modifications, respectively. Peptide identification statistics, including estimating random match probabilities and false discovery rates, were performed using a two-variable Gaussian model that discriminates between true and false hits [35] with some modifications as described elsewhere [36]. A false discovery rate cut-off of ~1% was set at unique peptide level. The ubiquitinated protein candidates were determined using spectral counting with parameters similar to those described by Franco et al [37]. Briefly, proteins were considered candidates when a minimum of two unique peptides, a minimum of three observed spectra, and at least two fold more spectra were present in UBD pull-downs compared to negative controls. Using these parameters no reverse sequences were observed at the protein level, indicating low false discovery with these relatively conservative criteria. The ubiquitination sites were not explicitly mapped because the samples were treated with iodoacetamide, which induces a modification that mimics the diglycine signature of ubiquitination [38]. Heat map and Pearson correlation between samples were plotted using DAnTE [39].

Results and Discussion

Ubiquitin-binding domain features

Despite the potential utility of UBDs, only a few studies have used a specific UBD to purify ubiquitination targets and all lack a systematic comparison and optimization of experimental conditions [27, 28]. Based on data from the literature, we first systematically compared binding characteristics of eight UBDs (Table 1) used in this study. Motifs that bind to ubiquitin, conserved amino acid residues, and UBD affinities from existing databases and literature [26, 40-50] are shown in Figure 1. ClustalW alignment of amino acid sequences (Figure 1A) reveals that even though each of these domains have divergent sequences, some have conserved regions at the MFP (Met-Phe-Pro) and LL (Leu-Leu) ubiquitin-binding motifs (indicated by the bars) along with some core hydrophobic amino acid residues. Low conservation has been proposed to enable UBD binding to a broad spectrum of mono- or polyubiquitin chains with different affinities and specificities [26, 40-50]. For instance, in Figure 1B, UBA2 domain of hHR23A protein, which is identical to the UBD from hHR23B, has a higher affinity and selectivity to K48-linked chains [42]. In contrast, UBA domains from Dsk2 and UQ1 appear to bind equally well to all different types of ubiquitination [26].

The affinity of UBD/ubiquitinated protein interaction is an important consideration in affinity purification experiments, as it is the key determinant for wash stringency and nonspecific protein removal [51]. While detergents, salts, and even denaturing agents can be used to eliminate non-specific binding of proteins to the beads, they can also impair the binding of weak specific interactions (dissociation constant, K_D in the μM range). Because

some of the UBDs exhibited moderately high affinities (K_D in the nM to low μ M range, Figure 1B), we hypothesized that detergents and salts could be used to decrease non-specific interactions without interfering with binding to the targeted proteins [51]. Thus, pull-down washes were performed in the presence of 1% Triton X-100 and up to 500 mM NaCl.

UBD affinity purifications and selectivities

The scheme for analyzing the ubiquitinated proteome of RAW 264.7 macrophages is depicted in Figure 2. RAW 264.7 macrophages lysates were first incubated with agarose beads coupled either to glycine (negative control) or to selected UBDs to perform affinity pull-down experiments. After four washes with buffer that contained both detergent and NaCl, the released proteins were rinsed with NH_4HCO_3 buffer. Bound proteins were eluted using Laemmli loading buffer, separated by SDS-PAGE, and then visualized by silver staining.

Figure 3A shows that the washing steps efficiently removed most of the contaminant proteins, as judged by the negative control lane; thus, eliminating the need to perform multiple-step purifications and avoiding the protein losses associated with them. As such, UBDs appear to be an interesting alternative to His-tagged ubiquitin purification by IMAC, which is well-known to have a high background of non-specific protein binding, especially in eukaryotic cells [52]. On the other hand, under the mild washing conditions employed for purification with UBD, some proteins associated with ubiquitinated proteins are expected to be captured [30].

Proteins captured by each of the UBDs exhibit similar protein profiles (Figure 3A), suggesting that each UBD in this study is capable of pulling-down the most abundant ubiquitinated proteins in cells and with similar efficiency. Despite similar profiles, the pull-downs using NBR1-UBA, 19S-5Sa-UIM, Dsk2-UBA, and UQ1-UBA enriched more proteins with higher molecular masses (>100 kDa) than the other UBDs, which may be attributed to more efficient pull-downs of polyubiquitinated proteins by these UBDs (Figure 3A). To verify this hypothesis, Western blot using anti-ubiquitin antibodies was performed, which confirmed that these UBDs precipitated more polyubiquitinated proteins than the others (Figure 3B). Interestingly, none of the UBDs were capable of precipitating free monomeric ubiquitin under the experimental conditions employed for this study. Furthermore, Dsk2-UBA and UQ1-UBA were able to capture proteins with lower molecular mass (< 50 kDa), which we presume are monoubiquitinated proteins (Figure 3B). Dsk2-UBA precipitated an ubiquitinated protein of ~ 16 kDa that we speculate as being a diubiquitin chain (Figure 3B).

Western blots using monoclonal antibodies to polyubiquitination at K48 and K63 were performed to investigate the selectivity of the UBDs towards specific linkages of ubiquitination. Note that antibodies against other polyubiquitination linkages are not commercially available. NBR1-UBA, Dsk2-UBA, and UQ1-UBA readily captured K48-polyubiquitinated proteins (Figure 3C), whereas only NBR1-UBA and Dsk2-UBA captured enough protein to be detected by anti-K63 antibodies (Figure 3D). Based on Western blot results, Dsk2-UBA appears to have the broadest specificity by binding to mono- and polyubiquitinated proteins at K48 and K63. NBR1 apparently has much higher affinity to poly-ubiquitinated proteins compared to mono-ubiquitinated targets. The ability of Dsk2-UBA and NBR1-UBA to pull-down proteins is consistent with surface plasmon resonance data from the literature (Figure 1B) [26].

UQ1-UBA bound to mono-ubiquitinated and poly-ubiquitination proteins at K48; however, Western blots indicated no enrichment of the K63 linkage. SPR data from the literature suggests that UQ1-UBA should bind with moderate to high affinities to the three

post-translational modification types (Figure 1B). The discrepancy could result from the Western blot being insufficiently sensitive to detect the K63 polyubiquitinated proteins captured by UQ1-UBA or that this type of polyubiquitination is not very abundant in RAW 264.7 cells. The K63 polyubiquitination band intensities were not very bright for the whole cell lysate, even after overexposing the Western blot (data not shown). Since K63 polyubiquitination is frequently associated with cell signaling, we speculate that this type of polyubiquitination may be very tightly regulated in macrophages and be upregulated during cellular activation with either cytokines or pathogen associated molecular patterns [9, 53]. Recently, the Fenselau group has developed a mass spectrometry method for sensitive confirmation of proteins polyubiquitinated with the K63 linkage that could be used combined with the UQ1-UBA to further investigate this apparent discrepancy with greater sensitivity [54].

Proteomic analysis of UBD-captured proteins

To date, large-scale ubiquitinated proteome analyses employing total cell lysates on the order of 10-100 mg for affinity purification followed by 2D LC-MS/MS have resulted in the identification of several hundreds to thousands of ubiquitinated proteins [17, 19, 32, 37, 55]. In this work, we employed a small-scale proteomic analysis approach using only 500 μ g total macrophage lysate for affinity purifications to demonstrate the efficiency of this strategy for sample-limited experiments, such as limited tissue sections. Following incubation with the lysate, the beads were extensively washed and proteins digested on-bead with trypsin (Figure 2). On-bead digestion avoids losses associated with the elution process and buffer exchange prior to digesting the proteins. A potential disadvantage is that on-bead digestion may contribute peptides derived from the digestion of the bait protein. However, as the UBDs are short in length, limited contamination from UBDs was expected, and only p62-UBA and UQ1-UBA contributed relatively high backgrounds (>200 spectral counts) per typical analysis (Table 1). Even the background from p62-UBA was not enough to interfere with the detection of ubiquitinated proteins since these samples also had the highest number of identifications (Figure 4).

Other UBDs, such as NUB1-UBA and 19S 5Sa-UIM, exhibited limited observation events, and hHR23B-UBA2 and NBR1-UBA were not observed. Additionally, Dsk2-UBA and VPS9-CUE sequences were not present in the searched sequence database. Moreover, since Dsk2-UBA and UQ1-UBA lack lysine residues, the use of a more specific enzyme, such as endoproteinase Lys-C would completely prevent the cleavage and release of peptides derived from these UBDs. The use of multiple UBDs in tandem, referred to as TUBE may increase the avidity to ubiquitinated proteins, leading to a more efficient capture of proteins [27, 28]. However, the size of TUBE compared to single UBDs would further complicate the complexity of mass spectrometry data. To circumvent this issue, Shi et al. separated the captured proteins using SDS-PAGE, which allowed the TUBE band to be analyzed separately [28].

Even though our proteome analysis consisted of single dimension LC-MS/MS, a number of ubiquitinated protein candidates, ranging from 154 to 217 proteins per UBD (Figure 4A) were found, which is comparable with other more extensive proteomics analyses that typically require an order of magnitude more sample [17, 19, 32, 37, 55]. The number of protein identifications per pull-down did not correlate well with the Western blot analysis, with one exception; that is, the spectral count for ubiquitin was directly proportional to the reactivity in the Western blot performed using anti-ubiquitin antibodies (Figures 3B and 4A, ubiquitin 1 spectra). The number of protein identifications did match with the SDS-PAGE protein profile. Expectedly, all UBDs were able to capture the most abundant ubiquitinated proteins (Figures 3B and 4A) [56-58] with similar efficiency, which were ribosomal, poly(A)-binding and heterogeneous nuclear ribonucleoproteins (Table S1). The correlation

plot in Figure 4B shows that the profiles of protein captured with NUB1-UBA, NBR1-UBA, 19S 5Sa-UIIM and VPS9-CUE are very similar, which may be due in part to the fact that they pull-down the same ubiquitinated targets. Conversely, the correlations of proteins purified with Dsk2-UBA, UQ1-UBA and NBR1-UBA, which had the highest reactivity with anti-ubiquitin antibodies, were not as high, suggesting that the combination of a few UBDs could improve the coverage of the ubiquitinated proteome.

Overall, the collective results of this study support the use of UBDs for ubiquitinated proteome analysis. It is also worth noting that these analyses can be readily scaled up and with the combination of more extensive separations, could lead to identification of much larger numbers of ubiquitinated proteins.

Conclusions

The use of UBDs to purify ubiquitinated proteins affords a simple alternative to tagged-ubiquitin and antibody approaches for analyzing the ubiquitinated proteome. UBD-based purification has the advantage of being applicable to small amounts of tissues and cells without the need to express exogenous DNA. In addition, this approach allows for digestion of the captured proteins on beads aiding parallelization and decreasing the losses associated with elution of proteins from the beads, further removing a high background of peptides derived from immunoglobulin chains. Although UBDs from p62, hHR23B, NUB1, 19S-5Sa and VPS9 exhibit low affinity (μM range) to ubiquitinated proteins, binding domains from NBR1, Dsk2 and UQ1 have reasonably high affinities (nM to low μM range) that enable purifications in more stringent conditions, thus reducing non-specific protein interactions to the beads. Based on data presented in this paper, we are using Dsk2-UBA in our current experiments, but the combination of UBDs from Dsk2, NBR1 and UQ1 might also be suitable to capture a broader range of ubiquitinated proteins.

As a perspective, the discovery and characterization of new short UBDs with high affinity to ubiquitinated proteins and some others with selectivity to certain types of polyubiquitination or monoubiquitination could represent novel potential reagents for more targeted pull-down assays. For example, Kaiser et al. used highly selective UBDs to capture and quantify pools of monomeric, polymeric, and monomeric-free ubiquitin from cells [59]. Elsewhere, recently developed antibodies that recognized signature diglycine residues remnant after tryptic digestion seem to be an interesting alternative for enriching peptides derived from ubiquitinated proteins [3, 4, 21] except that the signature diglycine residues are also present in proteins modified with other ubiquitin-like proteins, such as NEDD8 and ISG15. Thus, a combination of protein- and peptide-level enrichment might offer the best approach for future experiments with ubiquitinated proteomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Features of the ubiquitin-binding domains (UBDs) used in this study. **(a)** Sequence alignment of UBDs. The MFP and LL motifs that bind to ubiquitin are marked with bars, and the conserved amino acid residues are highlighted in grey and black. **(b)** Affinities of UBDs in terms of dissociation constants (K_D) retrieved from the literature (see Table 1 for references), which are highlighted as a heat map. ND: not determined. *The dissociation constant was determined to the UBA2 domain of hHR23A, but the sequence of this region is identical to the one from its paralog hHR23B.

Figure 2.

Ubiquitinated proteome analysis scheme for RAW 264.7 macrophages. Cells were lysed with detergent, and mono (Ub) and polyubiquitinated (PolyUb) proteins were captured with different UBDs coupled to agarose beads. The performance of UBDs to capture Ub and PolyUb proteins was evaluated using Western blot, or by on-bead digestion with trypsin followed by LC-MS/MS.

Figure 3. UBD-affinity purifications and Western blot analysis of RAW 264.7 macrophage cell lysates. Ubiquitinated proteins from macrophage lysates were pulled-down with each of the eight UBDs (p62, hHR23B, NUB1, NBR1, 19S-S5a, VPS9, Dsk2, and UQ1) or control beads and analyzed by SDS-PAGE followed by visualization with (a) silver staining or analyzed using Western blot and (b) anti-ubiquitin, (c) anti-polyubiquitination at K48, or (d) K63 antibodies. *represents free ubiquitin. #10 μ g of total lysate was loaded for the SDS-PAGE, whereas 100 μ g was used for Western blot.

Figure 4.

Proteomic analysis of ubiquitinated proteins obtained by affinity purifications with UBDs. (a) Spectral count heat map of identified proteins in the ubiquitination dataset. The heat map was constructed with only ubiquitinated protein candidates after removing contaminant proteins. The bottom of the graph shows the number of ubiquitinated protein candidates found in the pull-down performed using UBDs, along with the number of detected spectra for ubiquitin (between parenthesis). (b) Correlation plot of ubiquitinated protein candidates found within the purification of each UBD. Both heat map and correlation plot were produced using DANTE.

Table 1

Selected UBD names, characteristics and known functions.

Protein	Other names	Accession number	Domain	Region (residues)	Origin	Function	Ref
p62	Sequestosome 1	NP003891	UBA	387-436	Human	Autophagy	[43, 45, 50]
hHR23B	RAD23 homolog B	AAH20973	UBA2	360-409	Human	Proteasome degradation	[26, 42]
NUB1	NEDD8 ultimate buster 1, NUB1L	AAH446354	UBA	376-413	Human	Proteasome degradation of NEDD8-ylated proteins	[41]
NBR1	Next to BRCA1	Q14596	UBA	904-966	Human	Autophagy	[43]
19S-5Sa	19S subunit 5Sa of proteasome, Rpn10	NP002801	UIM	252-318*	Human	Proteasome degradation	[47, 49]
VPS9	Vacuolar protein sorting-associated protein 9	P54787	CUE	394-451	Yeast	Endocytic trafficking	[40, 46]
Dsk2p	Dsk2p	NP_014003	UBA	327-371	Yeast	Proteasome degradation	[47]
UQ1	Ubiquitin 1	AAH39294	UBA	517-588	Human	Protein quality control via proteasome/ERAD	[26, 44, 48]

*The region was not specified by the company; thus, for the alignment, 15 amino acid residues adjacent to each side were included.

Table 2
Spectral counts of bait-derived peptides

Bait	Spectral count (average per replicate)
p62-UBA	275.5
hHR23B-UBA	Not found
NUB1-UBA	39
NBR1-UBA	Not found
19S-5Sa-UIM	8
VPS9-CUE	Not found
Dsk2-UBA	Not found
UQ1-UBA	244.5